Expression of Hypoxia-related Tissue Factors Correlates with Diminished Survival of Adjuvantly Treated Patients with Chromosome 1p Aberrant Oligodendroglial Neoplasms and Therapeutic Implications

Peter Birner,[1,2] Matthias Preusser,[1] Ellen Gelpi,[1] Johannes Berger,[3] Brigitte Gatterbauer,[4] Inge M. Ambros,[5] Peter F. Ambros,[5] Till Acker,[6] Karl H. Plate,[6] Adrian L. Harris,[7] and Johannes A. Hainfellner[1]

Institutes of [1]Neurology, [2]Pathology, and [3]Brain Research and [4]Department of Neurosurgery, Medical University Vienna, Vienna, Austria; [5]Children Cancer Research Institute, St. Anna Hospital, Vienna, Austria; [6]Institute of Neurology (Edinger Institute), Johann Wolfgang Goethe University, Frankfurt, Germany; and [7]Cancer Research UK, Growth Factor Group, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom

ABSTRACT

Purpose: **Oligodendroglial neoplasms with chromosome 1p deletion are chemosensitive, and stratified adjuvant therapies have been proposed on the basis of 1p status. In this study, we evaluated expression of hypoxia-related factors and its influence on survival in oligodendroglial brain tumors with chromosome 1p aberrations.**

Experimental Design: **Forty-four primary and 16 recurrent oligodendroglial neoplasms with 1p aberrations (deletion or imbalance) were investigated immunohistochemically for expression of hypoxia-inducible factor 1α and carbonic anhydrase-9. We used *in situ* hybridization to investigate expression of vascular endothelial growth factor-mRNA. We defined as "low hypoxia score" expression of no or only one marker and as "high hypoxia score" expression of two or three markers. The predominant vascular patterns of tumors were defined as classic or bizarre vascular formations, based on anti-CD34–immunostaining.**

Results: **High hypoxia score was evident in 16 of 44 (36.4%) primary tumor specimens and in 14 of 16 (87.5%) recurrent tumors ($P = 0.001$). High hypoxia score was associated with the presence of bizarre vascular proliferations and WHO grade III. In the subgroup of patients who received adjuvant therapy, univariate analysis showed significantly shorter survival of patients with high hypoxia score ($n = 27$; $P = 0.0145$). For all of the primary tumors, hypoxia score was an independent prognostic factor ($P = 0.045$).**

Conclusions: **A fraction of oligodendroglial neoplasms with 1p aberrations shows evidence of tissue hypoxia, which significantly influences survival of patients receiving adjuvant therapy. Evaluation of tissue hypoxia could become useful for recruitment of patients for individualized therapy strategies, *e.g.*, selection of patients with hypoxic tumors for hyperbaric oxygenation preceding radiotherapy.**

INTRODUCTION

Oligodendroglial neoplasms are rare diffusely infiltrating primary brain tumors, standard therapy regimens comprise surgical resection and postoperative adjuvant therapy (1).

DNA-aberrations on chromosome 1p are characteristic features of oligodendroglial neoplasms (2), clinicopathological correlations showed significantly better response to adjuvant chemotherapy as compared with oligodendrogliomas with intact chromosome 1p (3). Stratified protocols on basis of 1p status for adjuvant therapy have been proposed and are evaluated in prospective clinical trials (4). The aim of these trials is to avoid radiotherapy and radiotherapy-induced adverse effects (such as cognitive decline and radionecroses) in patients with 1p deficient tumors. Although genetic differences between tumors are critical for outcome, interactions with the microenvironment provide an additional modifying factor. Because efficacy of radiotherapy is diminished in hypoxic tumor tissues (5, 6), tissue hypoxia may also influence response to adjuvant therapy in oligodendroglial neoplasms in addition to 1p status. However, systematic studies of tissue hypoxia in 1p aberrant oligodendroglial neoplasms are lacking thus far. The aim of this study was to evaluate evidence of tissue hypoxia and its impact on outcome in 1p aberrant oligodendroglial neoplasms.

MATERIALS AND METHODS

Patients. Routinely processed and paraffin-embedded tissue specimens of 44 primary (29 men and 15 women) and of 16 recurrent oligodendroglial tumors were included in this study. At resection of primary tumors, gross total resection of the tumor was possible in 19 cases, whereas only subtotal resection could be done in 23 cases. No information on radicality of resection was available in 16 patients. In 2 cases tissue was obtained at stereotactic biopsy. Histologic typing and grading was done according to WHO criteria (1). All of the speci-

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Requests for reprints: Johannes A. Hainfellner, Institute of Neurology, AKH 4J, POB 48, Währinger Gürtel 18-20, A-1097 Vienna, Austria. Phone: 43-1-40400-5507; Fax: 43-1-40400-5511; E-mail: Johannes.Hainfellner@meduniwien.ac.at.

Table 1 Primary *versus* recurrent tumors

Parameter	Primary tumors ($n = 44$)	Recurrent tumors ($n = 16$)
Histological typing		
Oligodendroglioma	30 (68.2%)	12 (75%)
Oligoastrocytoma	14 (31.8%)	4 (25%)
Histological grading (WHO)		
Grade II	34 (77.3%)	8 (50%)
Grade III	10 (22.7%)	8 (50%)
Chromosome 1p status		
Deletion	36 (81.8%)	14 (87.5%)
Imbalance	8 (18.2%)	2 (12.5%)
Vascular pattern		
Classic*	23 (52.3%)	3 (18.8%)
Bizarre*	21 (47.7%)	13 (81.2%)
Expression of proteins/mRNA†		
HIF-1α expression*	17 (38.6%)	13 (81.3%)
VEGF-mRNA expression	15 (34.1%)	9 (56.3%)
CA IX-expression*	19 (43.1%)	13 (81.3%)
Hypoxia score		
Low*	28 (63.6%)	2 (12.5%)
High*	16 (36.4%)	14 (87.5%)

Abbreviations: HIF-1α, hypoxia-inducible factor 1α; CA IX, carbonic anhydrase-9.

* Significant difference between primary and recurrent tumors (χ^2 test or Mann-Whitney test).

† "yes" or "no" principle.

mens had aberrations (deletion or imbalance status) of chromosome 1p (see Table 1; ref. 7). We based selection of cases on chromosome 1p status to study a homogenous tumor group and to avoid inclusion of clear cell primary brain tumors with different genetic status. Basic characterization of tumors used in this study is given in Table 1, tissues of these cases have also been used in previous studies (7–9). Mean age of patients with primary tumors was 44 ± 9.2 years. In addition to surgery, 18 patients received adjuvant radiotherapy, and 9 patients combined radiochemotherapy. Details on applied chemotherapeutic regimens were not available in this long-term retrospective study. No adjuvant therapy was administered to 13 patients, no information was available in 4 patients. Mean follow-up time was 60 months (range 1 to 150 months), during this observation time, 13 patients (29.5%) died from their disease.

Seven patients with recurrent tumors were male, and 9 patients with recurrent tumors were female. The mean age of patients at recurrence was 45.48 ± 7.6 years. Of 7 patients, tissue of primary tumors and recurrent tumors were available and used in the primary as well as in the recurrent tumor group.

Five patients (31.3%) with recurrent tumors received no adjuvant therapy for their primary tumors, 6 patients (37.5%) received adjuvant radiotherapy, 4 patients (25%) received adjuvant radiochemotherapy, and no data on adjuvant therapy preceding recurrence was available in 1 patient.

Analysis. Fluorescent *in situ* hybridization was done as described previously (7). Expression of hypoxia inducible factor 1α, carbonic anhydrase-9, vascular endothelial growth factor (VEGF), and CD34 proteins was determined on sections of formalin-fixed and paraffin-embedded tumor specimens by immunohistochemistry as described previously (10, 11). *In situ* hybridization VEGF and VEGF-receptor 1, with cRNA probes generated from cDNA clones containing the human *VEGF* or *FLT* gene, was done as described previously (12, 13).

A tumor was classified as positive for hypoxia-inducible factor-1α, VEGF-mRNA, or carbonic anhydrase-9 expression if at least five unequivocal positive tumor cells were found in the slide. Expression of no or only one marker was interpreted as evidence against significant tissue hypoxia and was designated as low hypoxia score. In contrast, expression of two or three markers was considered as evidence for significant tissue hypoxia (high hypoxia score).

Analyzing anti-CD34 immunostained sections, we defined vascular formations as described previously (10): (*a*) classic pattern of delicate branching capillaries (Fig. 1*A*); and (*b*) bizarre vascular formations (Fig. 1*B*) comprising glomeruloid vascular proliferations, vascular garlands, and vascular clusters. On the basis of these definitions, the vascular formations within a tumor were classified as predominantly "classic" or as predominantly "bizarre" according to previously reported criteria (10).

Statistics. Mann-Whitney-test and χ^2 test were used as appropriate. Overall survival was defined from the day of initial surgery until death of the patient. Death from another cause than oligodendroglial tumor and survival until the end of the observation period were considered as censoring events. Univariate analysis of overall survival was done as outlined by Kaplan and Meier. The Cox proportional hazards model was used for multivariate analysis of overall survival. Patient age, adjuvant therapy (yes *versus* no), hypoxia score, and histologic WHO grading were included into Cox regression. A two-tailed $P \leq 0.05$ was considered as significant.

RESULTS

Results of histologic and immunohistochemical analysis, mRNA *in situ* hybridization, and fluorescent *in situ* hybridization analysis are summarized in Table 1. Immunostaining of VEGF protein showed antibody binding on endothelial cells of bizarre vascular formations (Fig. 1*C*), which showed also VEGF-receptor 1 mRNA signals by *in situ* hybridization (data not shown).

VEGF-mRNA, hypoxia-inducible factor-1α, and carbonic anhydrase-9 expression showed focal accentuations and was found most commonly at the border of necrotic tissue areas (Fig. 1, *D–F*). Samples of fluorescent *in situ* hybridization analysis are shown as Fig. 1, *G* and *H*. Bizarre vascular formations were significantly more common in recurrent tumors compared with primary tumors (Table 1; $P = 0.037$, χ^2 test). A significant association of high hypoxia score with WHO grade III was found at analysis of all of the tumors ($P = 0.002$, chi-square test). High hypoxia score was significantly more common both in primary and recurrent tumors with bizarre vascular formations ($P = 0.001$ and $P = 0.025$, respectively, χ^2 test).

In the 7 patients where tissue from initial surgery and also from recurrency was available, hypoxia score did not change in any of these cases between primary and recurrent tumor (6 high; 1 low hypoxia score). No association of extent of resection with hypoxia score or survival was observed ($P > 0.05$).

Histologic grading had no significant influence on survival ($P = 0.0545$, log rank test). There was a significant association

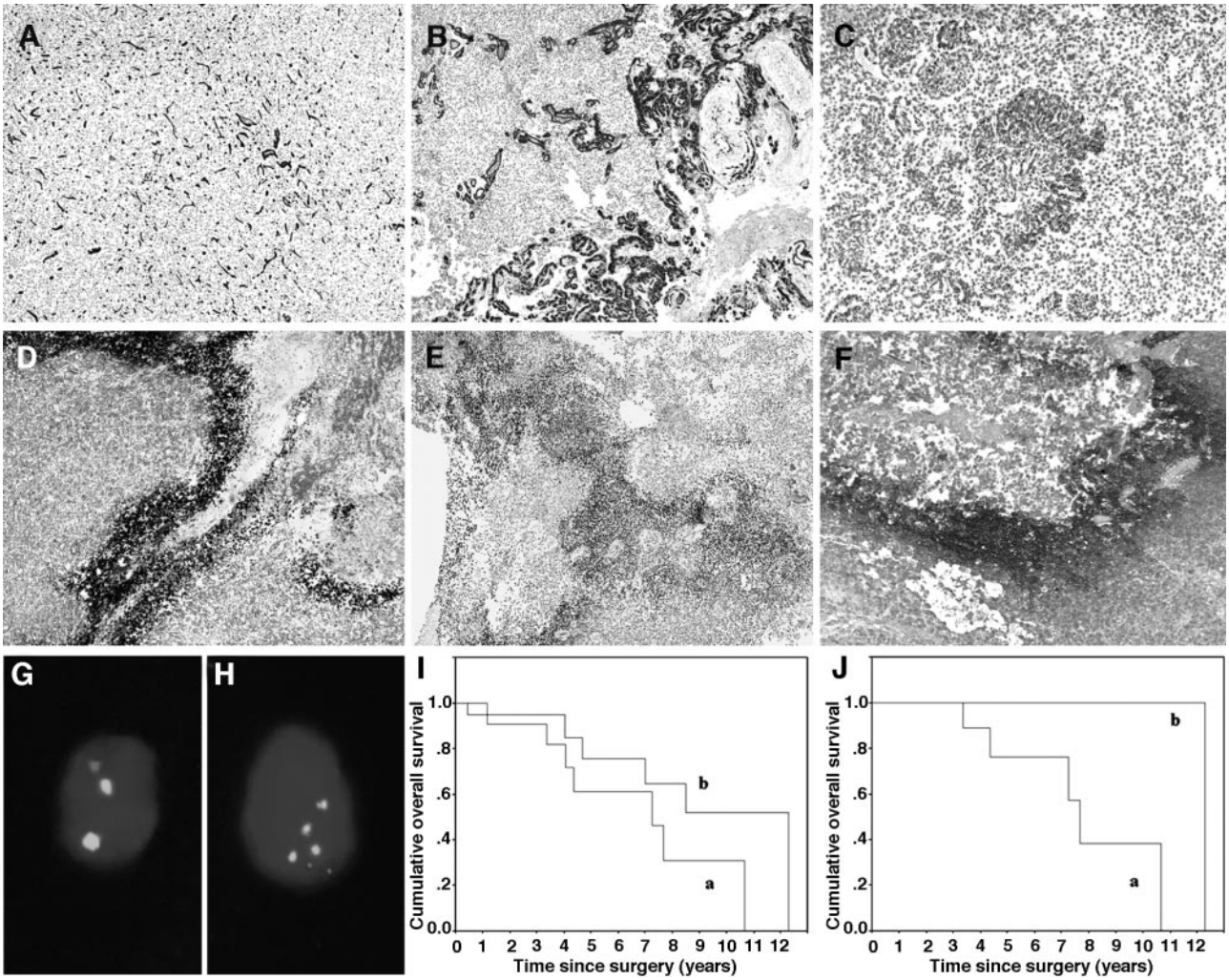

Fig. 1 *A*, classical angiogenic pattern–microvascular sprouting. This vascular pattern is characterized by an evenly distributed delicate capillary network (anti-CD34; ×100). *B*, bizarre angiogenic pattern with clustered, glomeruloid, and garland-like vascular formations (anti-CD34; ×100). *C*, anti-VEGF binding on endothelial cells of bizarre vascular formations (×200). *D*, VEGF-mRNA expression at the border of a necrotic tissue area assessed by *in situ* hybridization (×100). *E*, widespread expression of hypoxia-inducible factor 1α with patchy accentuations (antihypoxia-inducible factor-1α; ×40). *F*, prominent expression of carbonic anhydrase-9 at the border of a necrotic tissue area (anticarbonic anhydrase-9; ×200). *G*, chromosome 1p deletion status with a 2:1 ratio of paracentromeric *bright, large signals versus* subtelomeric *dim, small signals* (×3,000). *H*, chromosome 1p imbalance status with a 4:2 ratio of paracentromeric *bright, large signals versus* subtelomeric *dim, small signals* (×3,000). *I*, Kaplan-Meier curves of overall survival of all of the patients ($n = 44$) showing a clear trend toward diminished survival of patients with high tissue hypoxia score (log rank test, $P = 0.172$; Cox regression, $P = 0.045$). *J*, Kaplan-Meier curves of overall survival of patients who received adjuvant therapy ($n = 27$). Patients with high hypoxia score show significantly shorter postoperative survival times (log-rank test, $P = 0.0145$).

of adjuvant therapy with prolonged survival in univariate analysis ($P = 0.0033$, log-rank test).

Although expression of VEGF-mRNA, carbonic anhydrase-9, or hypoxia-inducible factor 1α showed no significant influence on survival of all of the patients in uni- and multivariate analysis, expression of each of these factors was associated with shorter overall survival in the subgroup of patients who received adjuvant therapy ($n = 27$) in univariate analysis ($P = 0.0191$, $P = 0.0352$, $P = 0.0086$, respectively, log-rank test).

A trend toward diminished survival in patients with tumors with high hypoxia score was found in Kaplan-Meier analysis (Fig. 1*I*), which missed significance ($P = 0.172$, log-rank test) in univariate but reached significance in multivariate analysis (see below). In the subgroup of patients who received no adjuvant therapy ($n = 13$), high hypoxia score (4 of 13 patients) had no influence on patient survival ($P = 0.1942$, log-rank test). In contrast, we observed in the group with adjuvant therapy ($n = 27$) a significantly shorter survival of patients whose tumors had a high hypoxia score (11 of 27 patients; $P = 0.0145$, log-rank test; Fig. 1*J*). There was no significant difference in survival time between the group of patients who received no adjuvant therapy ($n = 13$) and the group of patients who received adjuvant radiotherapy only and whose tumors had a high hypoxia score ($n = 6$; $P = 0.6494$, log-rank test). In contrast, we found a significantly shorter survival time in patients without adjuvant therapy ($n = 13$) than in patients who received com-

Table 2 Multivariate analysis of cumulative overall survival of 44 patients with primary oligodendroglial tumors (Cox regression)

Factor	*P*	Relative risk	95% interval of confidence
Adjuvant therapy (yes *versus* no)	0.004	0.023	0.002–69.049
Hypoxia score (low *versus* high)	0.045	9.53	1.053–86.253
Histological grading	0.155		
Patient age	0.707		

bined radiochemotherapy and whose tumors had a high hypoxia score ($n = 5$; $P = 0.0429$, log-rank test).

Adjuvant therapy and hypoxia score were independent prognostic factors of overall survival in multivariate analysis (Table 2).

DISCUSSION

In this study, we provide evidence that expression of hypoxia-related tissue factors correlates with diminished survival of patients with 1p-aberrant oligodendroglial neoplasms. We assessed expression of three different hypoxia-related factors according to a "yes" or "no" principle, and combined expression of 2 or 3 factors was considered as significant evidence for tissue hypoxia, because expression of only one factor may occur also in the absence of tissue hypoxia, *e.g.*, hypoxia-inducible factor-1α expression because of oncogenic activation. In a previous study, we considered hypoxia-inducible factor 1α expression in oligodendroglial tumors primarily because of oncogenic activation (8), but in the light of our current data, it seems now more likely that hypoxia-inducible factor 1α expression in oligodendroglial neoplasms is mainly induced by tissue hypoxia.

We also studied vascular patterns with anti-CD34 immunostaining and observed a spectrum of vascular formations in oligodendroglial tumors as described previously in glioblastoma (10). Most notably, bizarre vascular patterns, comprising glomeruloid vascular formations and vascular garlands, were observed and significantly more common in recurrent tumors. Furthermore, presence of bizarre vascular formations correlated significantly with high hypoxia score both in primary and recurrent tumors. These observations indicate that formation of bizarre vascular proliferations is linked to tissue hypoxia. Expression of hypoxia-related tissue factors may promote growth of bizarre vascular formations. Existence of such a mechanism is supported by expression of VEGF-receptor 1 in endothelial cells of bizarre vascular formations and concomitant immunohistochemical detection of VEGF protein on these endothelial cells.

However in this type of study, it is not possible do determine whether hypoxia generated the transcription program that induced bizarre vascular formations or whether such structures are particularly poor at delivering blood and hence cause hypoxia.

Survival analysis confirmed adjuvant therapy as a highly significant factor for outcome of patients with 1p-aberrant oligodendroglial neoplasms. In addition, survival analysis showed an inverse correlation of high hypoxia score with patient survival. Although in the whole patient group this correlation was visible in univariate analysis only as a trend, it was significant in the patient group with adjuvant therapy. In the whole collective, hypoxia score was an independent prognostic factor in multivariate analysis. Our data provide indirect evidence that tissue hypoxia influences efficacy of adjuvant therapy. In particular, chemotherapy seems to be decisive for a beneficial therapeutic effect in hypoxic tumors: there was no significant difference in survival time between patients who received no adjuvant therapy and patients who received adjuvant radiotherapy only and whose tumors had a high hypoxia score. In contrast, we found a significantly shorter survival time in patients without adjuvant therapy than in patients who received combined radiochemotherapy and whose tumors had a high hypoxia score. Although it was shown previously that in oligodendrogliomas 1p aberrations themselves seem to be associated with better response to adjuvant therapy compared with tumors with intact 1p (14), our data show that there are also differences within the group of 1p-deficient tumors with regard to response to adjuvant therapy because of the amount of tissue hypoxia.

To make tissue hypoxia scoring on routinely processed tissue samples amenable for clinical use, its correlation with *in vivo* measured tissue oxygen levels needs to be analyzed, *e.g.*, by the use of Eppendorf electrodes (15–17) or functional imaging techniques (18, 19). If tissue-based hypoxia scoring can be verified as indicator of tissue oxygen levels, it could become useful for recruitment of patients for individualized therapies, *e.g.*, identification of hypoxic tumors for hyperbaric oxygenation preceding radiotherapy (20). As a high percentage of oligodendroglioma recurrences in the 1p aberration setting have a high hypoxia score, it should be considered to include routinely strategies of tissue oxygenation in this patient subpopulation in case of adjuvant treatment.

Research on oligodendroglial tumors is generally complicated because of their rarity. So our results should be confirmed in large-scale, multicenter prospective studies. Additionally, the impact of tissue oxygenation should also be investigated in other adjuvantly treated brain tumor types, as hypoxia-induced resistance to radiotherapy may not be restricted to oligodendroglial tumors with chromosome 1p aberration.

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